Direct Visualization of Individual mRNA Export Through Differential Fluorescent Labeling

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Direct Visualization of Individual mRNA Export Through Differential Fluorescent Labeling

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Abstract

The nuclear export of mRNA through the nuclear pore complex is a crucial step in gene expression and is tightly regulated through signal-recognizing proteins. Though the mRNAs in the cytoplasm are well imaged in fixed or living cells, the detection of the export event has been difficult due to the lack of adequate technique to distinguish nuclear and cytoplasmic mRNAs, while it is crucial in elucidating the mechanism of controlling their life cycle. We developed a novel fluorescent labeling tool that will mark the nuclear and cytoplasmic mRNAs differently. Using the tool, we can potentially trace the full life cycle of mRNAs from transcription, splicing, to translation. We present our preliminary results on developing the labeling tool and tracking the live mRNA export dynamics.

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Nomenclature

cDNA: complementary DNA

FLP: Flippase

FP: Fluorescent Protein

FRT: Flippase Recognition Target

HR: Homologous Recombination

IPMK: Inositol Polyphosphate Multikinase

MCP: MS2 Coat Protein

MFE: Minimum Free Energy

NES: Nuclear Export Signal

NLS: nuclear Localization Signal

NPC: Nuclear Pore Complex

PCP: PP7 Coat Protein

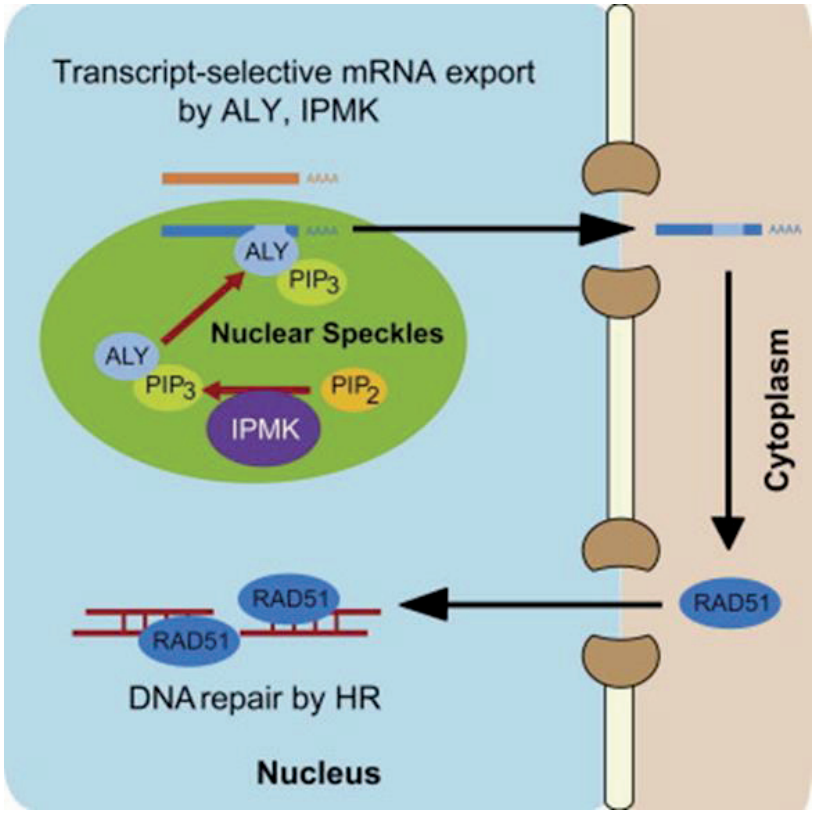
1. Introduction

A nascent mRNA is transcribed on the site of activating gene and form a mature mRNP after getting through splicing and processing. Transcription-export complex (TREX and TREX2) forms an exporting component with mRNA and translocates mRNA through nuclear pore complex (NPC) after docking on NPC. Although mRNA had been thought to be exported through the bulk export pathways, recent studies have indicated that nuclear export of mammalian mRNA is highly selective and can regulate essential biological processes such as gene expression, DNA repair and cell survival.

1.1. Control of mammalian gene expression by selective nuclear mRNA export

Nuclear mRNA export is mediated by conserved transport factors such as nuclear RNA export factor 1 (NXF1) and its cofactor p15, and a majority of mRNA utilize the NXF1-p15 dimer to get out of the nucleus. Mature mRNA interacts with NXF1-p15 dimer and TREX, which have different subset depending on the type of mRNA. For instance, transcripts translated protein involved in genome integrity interact with TREX including IPMK and ALY proteins.

To study the selective mRNA nuclear export in live cell, novel mRNA export imaging system is required for distinguishing nuclear and cytoplasmic mRNA. The differential fluorescent labeling system enables to differentiate the nuclear and cytoplasmic mRNA and quantify single mRNA. Although the transient transfection is a method expressing exogenous gene, we are going to be able to do endogenous gene tagging using CRIPR-Cas9 system after stabilizing this differential fluorescent labeling system.



Vihandha O. Wickramasinghe et al., Nature Reviews Molecular Cell Biology 16, 431–442 (2015)

Figure1. RAD51 has transcript-selective nuclear export pathway controlled by IPMK.

RAD51 mRNA export pathway is one of the representative selective pathway controlled by human inositol polyphosphate multikinase (IPMK) which has activity converting PIP2 to PIP3. ALY (RNA export factor) recognizes target transcripts in the presence of PIP3 converted by IPMK and mediates their export from the nucleus to the cytoplasm through nuclear pore complex (NPC). The selectively exported transcripts are translated to RAD51 proteins, and these proteins are transported again into the nucleus through NPC. RAD51 protein has a crucial role in homologous recombination (HR) during DNA double-strand break. Because export of RAD51 mRNA is tightly regulated, it is essential to study the mechanism of transcript-selective nuclear export. We expect to visualize direct nuclear export of individual RAD51 mRNA through differential fluorescent labeling.

1.2. Visualizing Single mRNA in live mammalian cell

Although RNA imaging tools are well utilized in fixed cell such as in situ hybridization, this method just shows a snapshot among the lifecycle of RNA at the certain time. On the other hand, RNA imaging in live cell enables to explore mRNA distribution and full life cycle of mRNA in real time, and A lot of RNA imaging system in a live cell has been developed. In MS2 and its similar system, one of the representative system to image RNA in a live cell, aptamer sequence is inserted in the middle of mRNA, and its coat protein fused to fluorescent protein recognize the RNA aptamer sequence. With this system, mRNA inserted MS2 sequence can be detected by the coat protein fused to FP and shown as a single spot. We designed MS2-PP7 alternating design and imaged live cell imaging using wide-field fluorescence microscopy.

Some of research group have studied mRNA nuclear export and imaged direct mRNA nuclear export by labeling mRNA and nuclear pore with different fluorescent color. However, it was difficult to quantify the number of mRNAs and distinguish the cytoplasmic and nuclear region because cell has three-dimensional figure. Even though cell has cytoplasmic area on the nucleus, it is undistinguishable when using especially wide-field microscope because it gives images with thick Z range. Therefore, we developed differential fluorescent labeling mRNA imaging system to determine the location of mRNA by its fluorescence color using dual MS2-PP7 tagging system.

2. Materials and Method

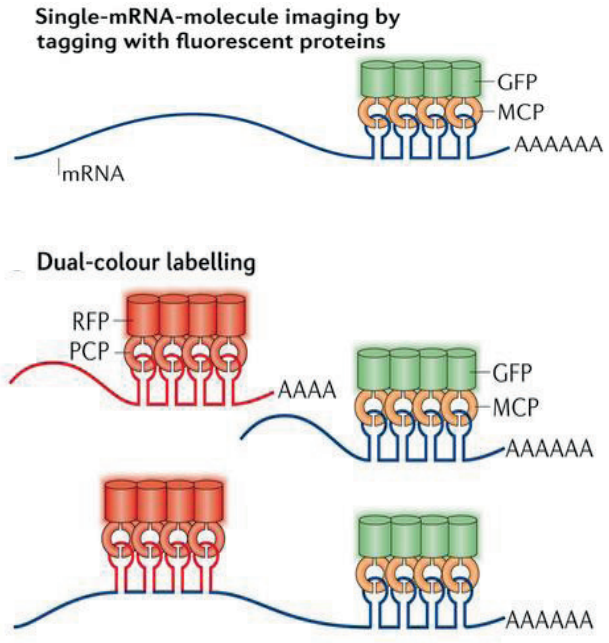
2.1 Cell Culture and Transient Transfection

Human HEK293 (from Korea cell bank), U-2 OS cells were grown in high glucose DMEM with 10% fetal bovin serum and 5% of penicillin/streptomycin in a humidified 5% CO2 incubator at 37 degrees. Cells were passaged every 3 or 4 days with Trypsin-EDTA (Thermo Fisher scientific) and transiently transfected with Lipofectamin2000 (Thermo Fisher scientific) and Opti-MEM reduced serum medium.

2.2 Fluorescence microscope

Wide-field fluorescence images were acquired using Olympus cell^R system with 60X or 100X oil immersion objectives. For the time-lapse imaging in living cell, cell samples are prepared on Confocal dishes (SPL) – glass-bottomed dish - and kept in the humidified chamber maintaining degrees at 37 and CO2 concentration. Confocal images were made with Zeiss LSM 880 using 63X oil immersion objective.

2.3 MS2, PP7 RNA tagging system



Adina R. Buxbaum et al., Nature Reviews Molecular Cell Biology 16, 95–109 (2015)

Figure2. MS2 and PP7 RNA tagging system to explore single mRNA

Imaging single mRNA in a live cell can be done by inserting MS2-like stem-loop sequence in a gene of interest and tagging Fluorescent protein to its RNA binding protein. For example, MS2 coat protein tagging to GFP recognizes MS2 stem-loop sequence. In dual-color labeling, two different RNA aptamer structures are tagged in one gene of interest by tagging its coat protein in different colors so that a mRNA is labeled by two distinct colors. Gene of interest is confirmed by colocalization of two distinct fluorescent signals in the same spot.

2.4 plasmid construction

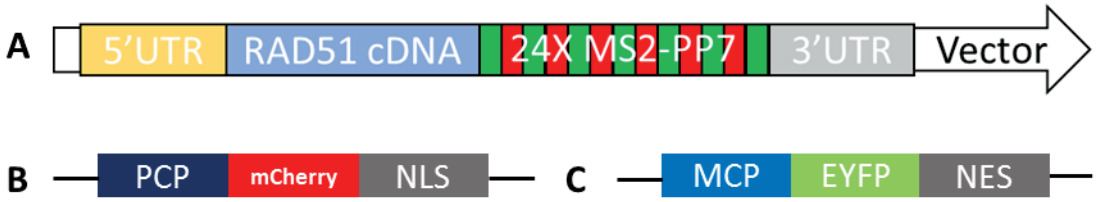


Figure3. Plasmid design of gene of interest inserted MS2-PP7 sequence and its coat protein

Three plasmid structures are designed to express a gene of interest inserted 24X MS2-PP7 sequence and its coat protein fused to fluorescent protein in mammalian cells. RAD51 gene plasmid is made by reverse transcription-PCR from endogenous mRNA including 3' and 5' UTR (untranslational region) and inserted 24X MS2-PP7 RNA stem-loop sequence designed in our lab. MS2-PP7 RNA aptamers are arrayed in an alternating manner instead of tandemly arranging each MS2 and PP7 RNA aptamer. The alternating 24X MS2-PP7 sequence is located between cDNA and 3'UTR of RAD51 gene to avoid post-transcriptional regulation. Post-transcriptional regulation such as shRNA or miRNA binds on 3'UTR to regulate gene expression at RNA level.

Coat proteins are fused to a fluorescent protein and nuclear localization signal (NLS) or nuclear export signal (NES) to make differential fluorescent labeling. PP7 coat protein fused to mCherry and NLS is transported into the nucleus, and MS2 coat protein fused to EYFP and NES is carried to the cytoplasm by cargo protein. Each coat protein detects its corresponding RNA aptamer (MS2-MCP and PP7-PCP), therefore nuclear and cytoplasmic mRNA can be labeled by distinct color using differential fluorescent labeling system.

2.5 stable cell line construction

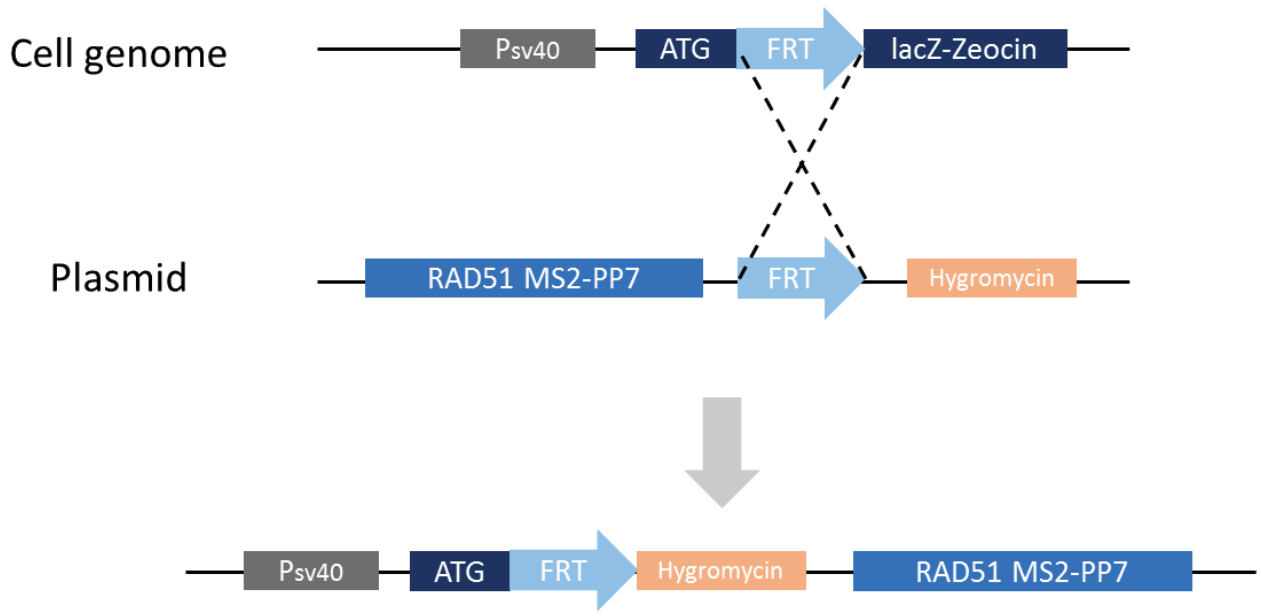


Figure4. Brief schematic image of Flp-in system to make stable cell line

Flippase(Flp) recognizes 13-bp 5'-GAAGTTCCTATTC-3' called flippase recognition target(FRT) and cleaves between the two identical FRT. A T-REx cell line selected with Zeocin and β-galactosidase activity assay and has FRT and promoter. We inserted RAD51-12X MS2-PP7 gene in the backbone plasmid which has FRT and Hygromycin gene for cell selection. Hygromycin protein is translated only when the plasmid construction has been inserted because hygromycin gene in the plasmid doesn't have start codon. The plasmid is cotransfected with a plasmid encoding Flp in the cell line having FRT in the genome, and the recombination can occur between two identical FRT. The newly made cell line express the gene of interest and hygromycin resistance protein so that it enables selection with medium containing hygromycin antibiotics.

3. Results

3.1 Expression of plasmid in mammalian cells

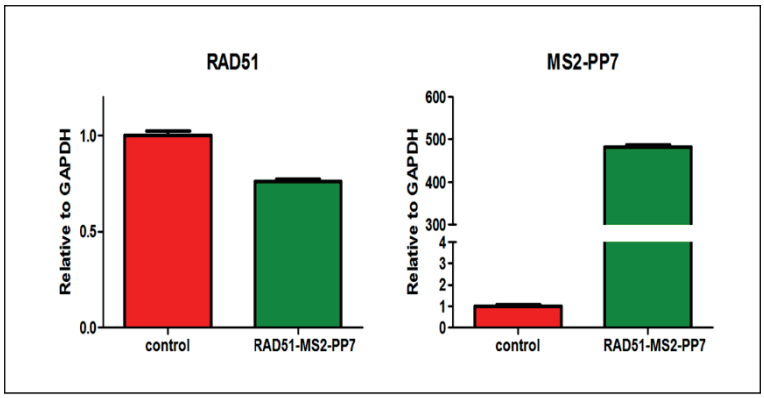


Figure5. Real-time qPCR results of the stable cell line expressing RAD51 inserted MS2-PP7

To investigate the expression of RAD51 mRNA inserted alternating MS2-PP7 sequence, real-time PCR is used for quantifying amount of RAD51 mRNA and alternating MS2-PP7 repeat sequence. RAD51 mRNA sequence is similarly expressed in the stable cell line whereas alternating MS2-PP7 sequence is high expressed in the only stable cell line. Through this real time qPCR result of the stable cell line, we can confirm that the stable cell line is sustainably expressing RAD51 mRNA tagging MS2-PP7 alternating sequence, and besides MS2-PP7 RNA sequence doesn't affect to RAD51 expression level.

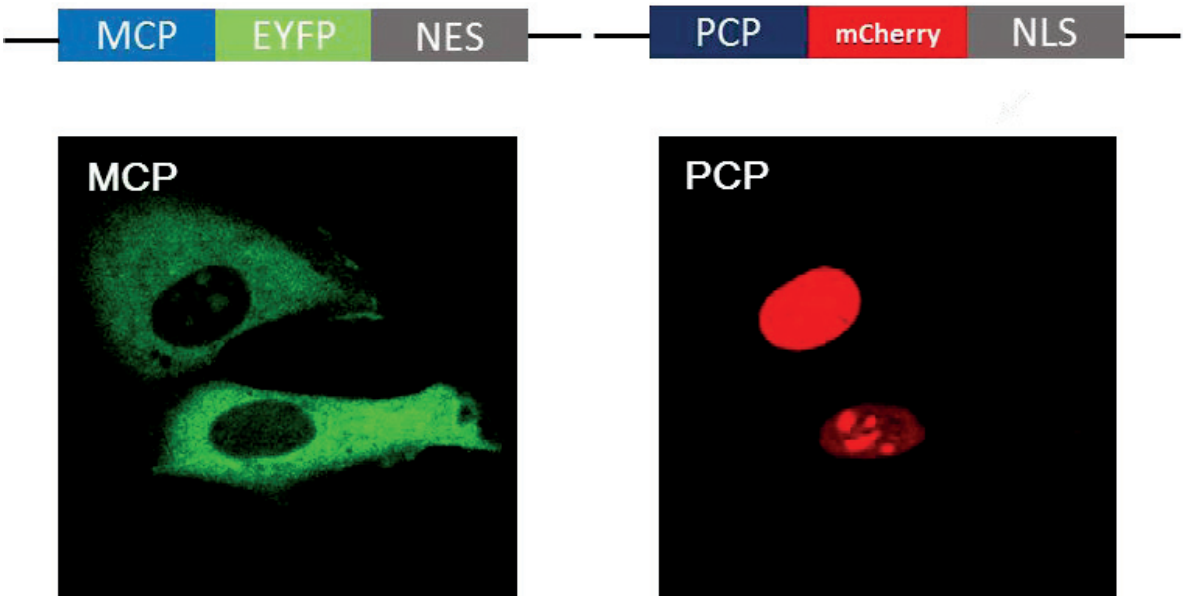


Figure6. Differential fluorescent Labeling in mammalian cell

U-2 OS cells are transiently transfected with two plasmids MS2 coat protein-EYFP-Nuclear export signal (MCP-EYFP-NES) and PP7 coat protein-mCherry-Nuclear localization signal (PCP-mCherry-NLS) to distinguish the nucleus and cytoplasm by differential fluorescent labeling. Cargo proteins recognize NES or NLS and transport the protein with NES or NLS to cytoplasm or nucleus respectively. After the plasmids are translated into proteins, the PCP-mCherry-NLS is actively transported into the nucleus and MCP-EYFP-NES is kept staying just in the cytoplasm and not transporting into the nucleus. In a cell, nucleus and cytoplasm are labeled by differential fluorescent and nuclear and cytoplasmic RNA are identified by different color.

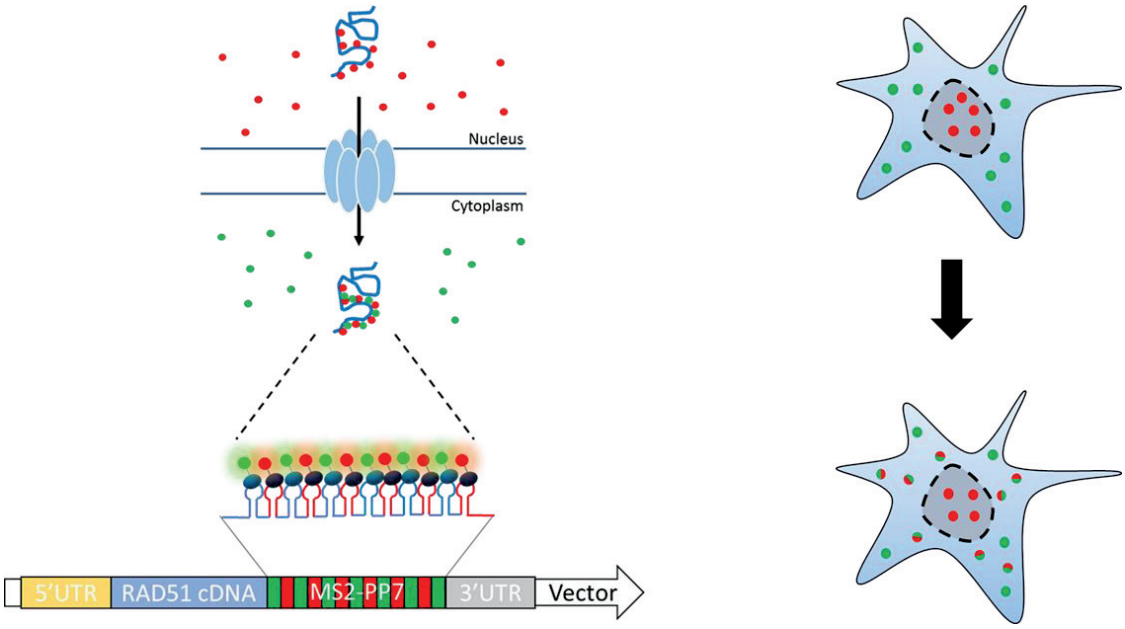


Figure7. Schematic workflow of differential fluorescent labeling

Schematic image shows how the differential fluorescent labeling system works for labeling nucleus and cytoplasm and distinguishing the nuclear and cytoplasmic RAD51 mRNA in a cell. RAD51 mRNA inserted MS2-PP7 sequence is transcribed in the nucleus, and the transcribing RAD51 mRNA can be detected as a red spot with PCP-mCherry-NLS binding on the PP7 RNA aptamer in the RAD51 mRNA. After the mature mRNA is exported into the cytoplasm, the cytoplasmic RAD51 mRNA is identified as a colocalization of red and green spots in the cytoplasm. Therefore, the mRNA export event can be effectively captured with color change and spot colocalization in two channels using differential fluorescent labeling.

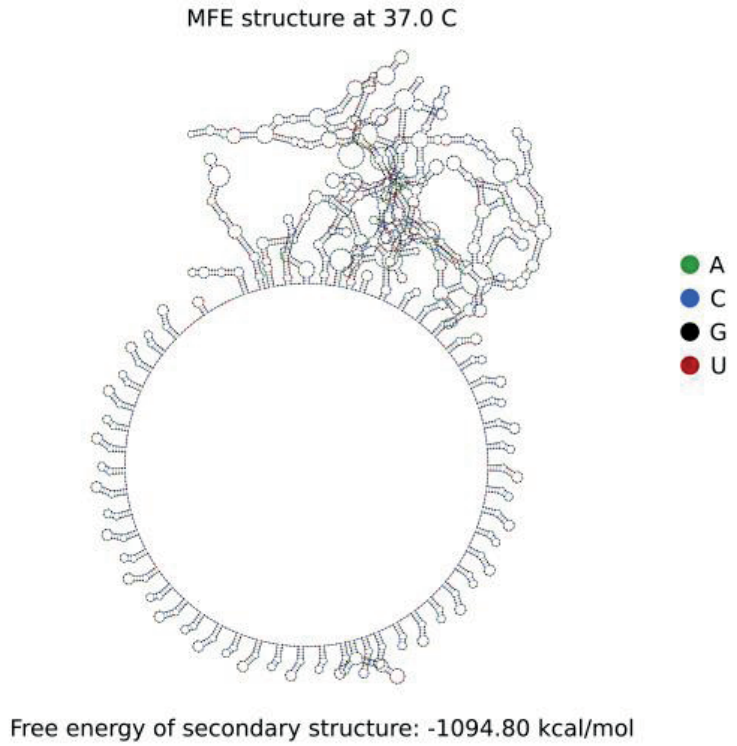
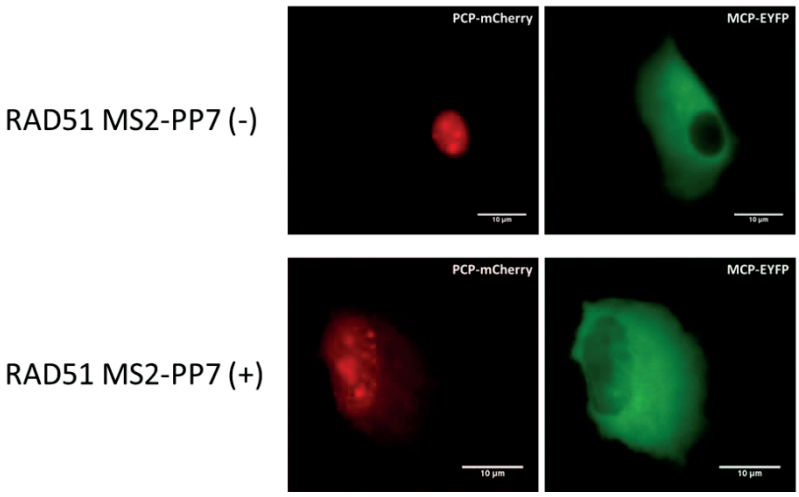


Figure8. Anticipated secondary structure of RAD51 inserted 24X MS2-PP7 RNA stem-loop structure by NUPACK

NUPACK (Nucleic Acid Packaging) is a suitable software for predicting and designing nucleic acid secondary structure. The anticipated secondary structure of RAD51 mRNA inserted 24X alternating MS2-PP7 aptamer is predicted at 37 degrees by the program. The stem-loop structures are well conserved after inserting in the RAD51 gene.



$$\frac{I_{Cytoplasm} - I_{Background}}{\{(I_{Nucleus} - I_{Background}) + (I_{Cytoplasm} - I_{Background})\}}$$

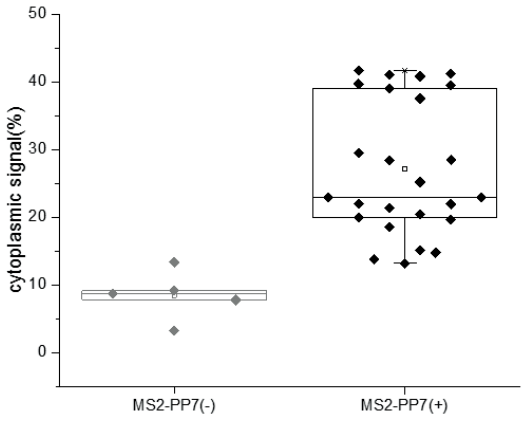


Figure9. PCP-mCherry nuclear co-export with RAD51 MS2-PP7 mRNA.

Even though a PCP-mCherry-NLS is delivered into the nucleus because of nuclear localization signal at the end of the protein, cells show cytoplasmic PCP-mCherry signal only when expressing RAD51 MS2-PP7 mRNA. The PCP-mCherry recognize PP7 RNA aptamer on the RAD51 MS2-PP7, and is co-exported with the mRNA to the cytoplasm. We analyzed how much the cytoplasmic RAD51 MS2-PP7 signal has been increased compared to cells non-expressing MS2-PP7 RNA (n=25) The cells expressing RAD51 MS2-PP7 mRNA have distribution from 10% to 40% of cytoplasmic signal whereas non-expressing cells show nearly less than 10% of cytoplasm signal.

3.2 Transcription site of RAD51 gene

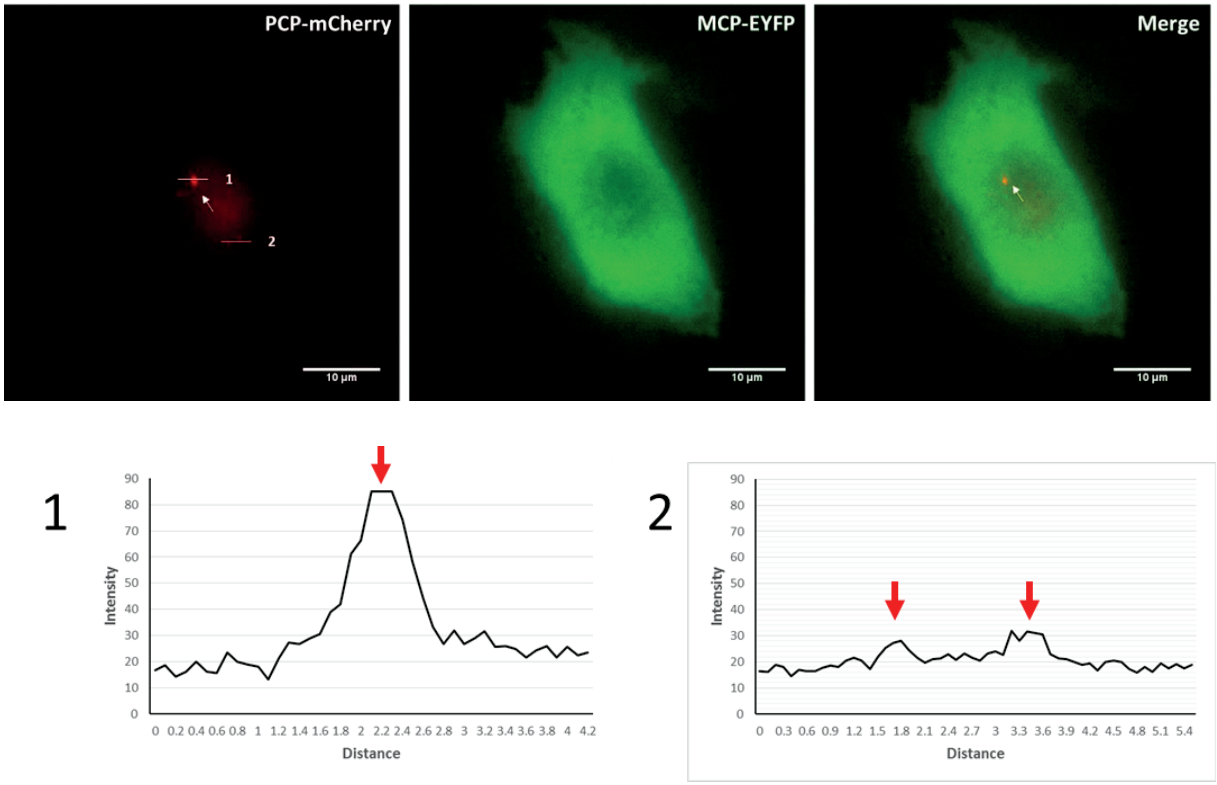


Figure10. Florescent intensity comparison between transcription site and single free mRNA

At the transcription site, several copies of mRNA are actively transcribed continuously. The PCP-mCherry binds on the transcribing mRNA, and the mRNAs are anchored nearby the site, therefore it appears as a brighter and bigger single spot than free mRNA spots. We compared the fluorescent intensity of transcription site and single free mRNA. The spot of transcription site has 3-fold higher intensity than free single mRNA. By comparing the intensity of the spots, we can estimate that 3 copies of mRNAs are on the transcription site.

3.3 Diffusion over a distance

$$< x^2 > = q_i D t$$

D - diffusion coefficient ($cm^2\ s^{-1}$)

$<x^2>$ - mean-square displacement

q_i - numerical constant which depends on dimensionality
q_i = 2, 4, or 6, for 1, 2, or 3 dimensional diffusion

t - time

Figure11. The valid equation for diffusion over a distance

To analyze mRNA mobility, we use valid equation for diffusion over distance. All trajectories drawn over time, and we choose the spots which have more than 30 frames and are distinguishable with eye. The X, Y position data is extracted from trajectory data, and mean square displacement is calculated from position dara. The q_i is defined as 4 because we take 2 dimensional images, and time interval is given from 5~10sec.

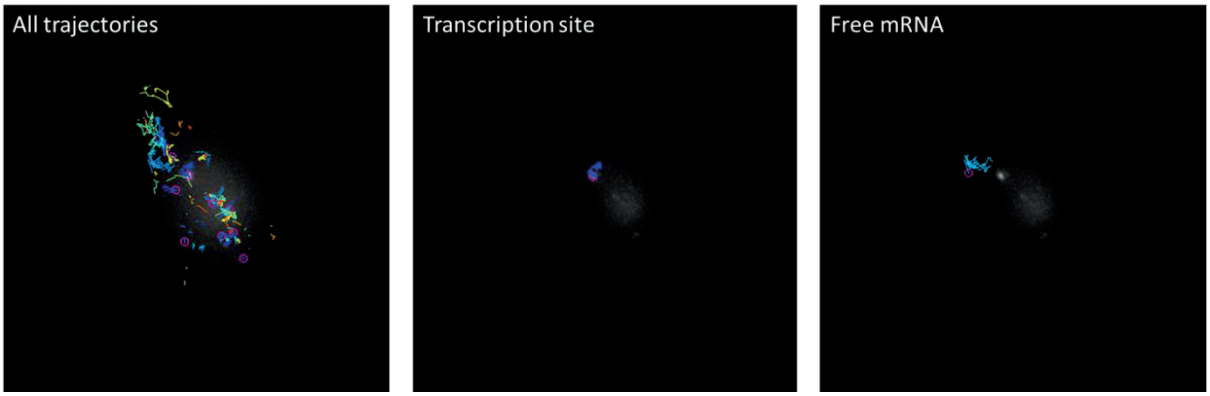


Figure12. Trajectories of all mRNAs in a single cell, transcription site and single free mRNA.

All trajectories in a single cell has been drawn in one frame, and we choose proper trajectories to compare its molecular activity by diffusion coefficient. The diffusion coefficient of the spot of transcription site and free single mRNA has been measured. The spot of transcription site have relatively low value than free mRNA spot. Exogenous RAD51 is incorporated into the cell genome and express RAD51 mRNAs continuously, so it has low diffusion coefficient than free mRNA as we expected.

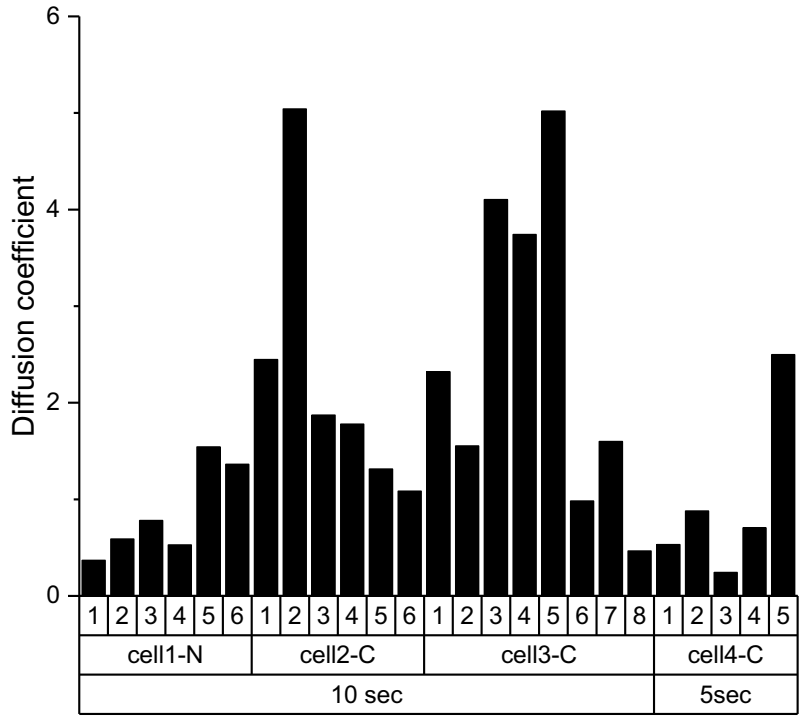


Figure13. RNA has various mobility (N-nucleus, C-cytoplasm)

A single cell has RNA molecules which show diverse motion in the cytoplasm and nucleus. Some of mRNAs with nearly zero diffusion coefficient show static mobility. The mRNA molecules with high diffusion coefficient have nearly 10-fold higher mobility than static mode and are actively transported by motor proteins such as kinesin or dynein. Lastly, others with moderate diffusion efficient show diffusive RNA mobility.

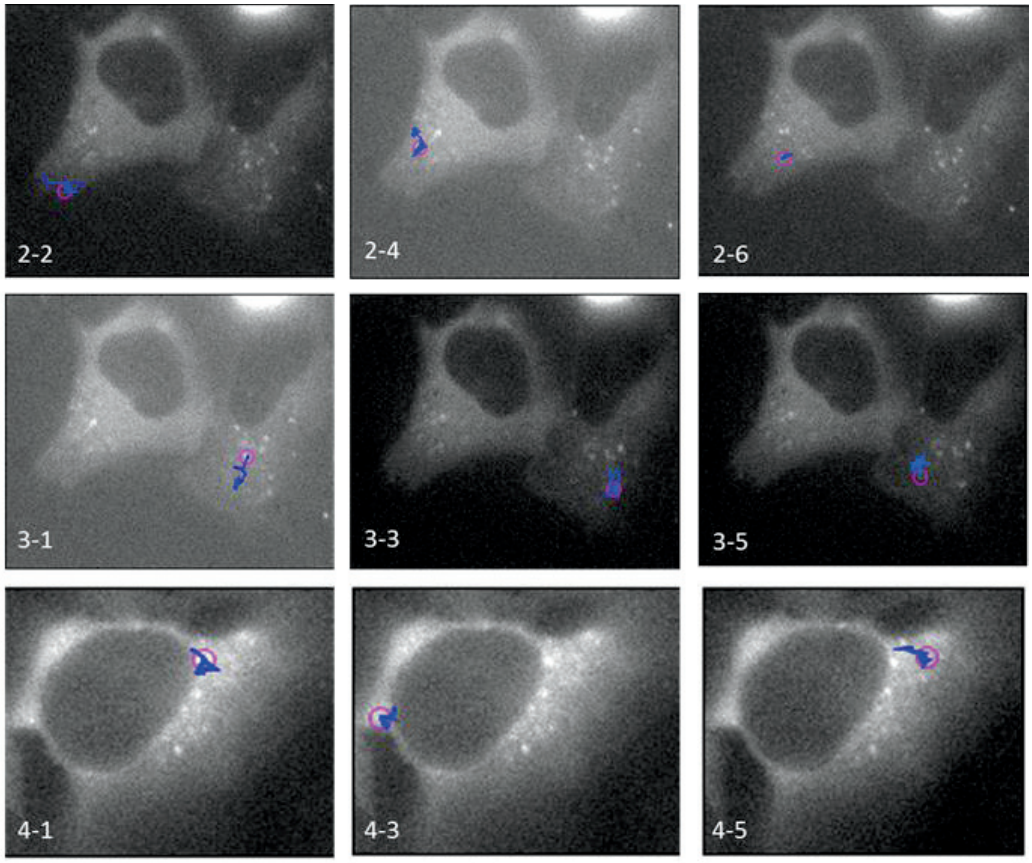


Figure14. Cell to cell variation of mRNA mobility and its trajectories.

3.4 Nuclear export of RAD51 mRNA

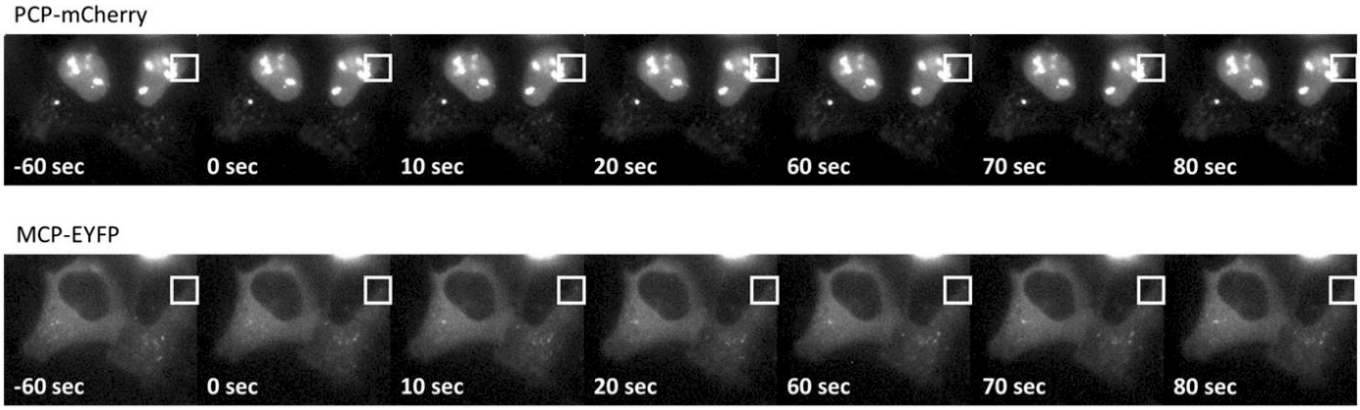


Figure15. Direct visualization of RAD51 mRNA export from the nucleus to the cytoplasm

Nuclear and cytoplasmic region are labeled with PCP-mCherry and MCP-EYFP respectively. Nuclear mRNAs are visulalized as red spot, and cytoplasmic mRNAs are distinguished from nuclear mRNAs by the colocalization of red and green spot. White rectangular region shows the direct RAD51 mRNA export event over time. Although it might be not exact because of somewhat long time interval (10 sec), a spot in both red and green channel appears at time 0 sec and disappears at the 80sec frame.

4. Discussion & Conclusion

The differential fluorescent labeling system is a powerful tool for distinguishing nuclear and cytoplasmic mRNA, quantifying the number of mRNAs and direct visualizing nuclear export of individual mRNA. Individual mRNA is visualized both in the nucleus and cytoplasm with PP7-PCP and MS2-MCP RNA tagging system respectively.

Even though some cells doesn't show single mRNA as a single spot, they have nearly 4-fold increased cytoplasmic PCP-mCherry signal compared to non-expressing RAD51 MS2-PP7 cells. Moreover, dynamics of mRNAs is analyzed from time-lapse differential fluorescent labeling system. From the diffusion coefficient data, free mRNAs have various type of mobility such as diffusive or actively transported mobility whereas transcription site shows nearly static movement. Lastly, direct RAD51 mRNA nuclear export event is captured by the changing color of RAD51 mRNA spot.

Although many interesting data of mRNA lifecycle has been identified through differential fluorescent labeling system, we still have some limitations to overcome. First of all, endogenous RAD51 mRNA might have different lifecycle and dynamics because RAD51 MS2-PP7 mRNA is incorporated in the cell genome exogenously at this study, and besides MCP and PCP binding on the mRNA may have affected to the dynamics of RAD51 mRNA. Moreover we need to get more quantitative data to analyze mRNA export dynamics and quantify respective the number of spot in the nucleus and cytoplasm.

For further study, we are planning to do followings: (1) Constructing all-in-one plasmid for fluorescent labeling proteins and stable cell line expressing exogenous 24X MS2-PP7 RAD51 mRNA (2) Quantifying the number of mRNAs in the nucleus and cytoplasm respectively. (3) Control experiments related to transcript-selective nuclear mRNA export (4) Stable cell line is going to be made which have MS2-PP7 tag right after endogenous RAD51 mRNA gene in the genome using CRISPR/Cas9 system to study endogenous mRNA.

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어느 새 2 년의 석사 학위를 마치며 졸업 논문을 제출하게 되었습니다. 이렇게 학위 논문을 완성하기 까지 여러분의 도움이 있었기에 이 공간을 빌려 감사의 말씀을 전하고자 합니다. 무엇보다도 항상 연구에 대한 열정의 조언과 격려로 지도해 주신 김하진 교수님과 바쁘신 중에도 시간을 내주신 이자일 교수님과 이현우 교수님께도 감사합니다.

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마지막으로 뒤에서 묵묵히 저를 지원해준 엄마, 아빠, 학진이와 힘들 때마다 항상 위로가 되어준 유은이에게도 감사합니다. 제가 앞으로 걸어 갈 길은 제가 지금까지 걸어 온 길보다 더 험난할 걸로 압니다. 우연히 학위 논문을 펴보았을 때, 목표와 열정을 가지고 학위 논문을 완성한 지금을 되새기며 한 발짝 더 나아갈 수 있는 제가 되겠습니다.